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# Nicotine Suppresses Tunicamycin-Induced, But Not Thapsigargin-Induced, Expression of GRP78 during ER Stress-Mediated Apoptosis in PC12 Cells

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**We previously reported that nicotine protected against tunicamycin (Tm)-induced ER stress-mediated apoptosis, but not thapsigargin (Tg)-induced apoptosis in PC12 cells. In the present study, we report that the expression of glucose-regulated protein 78 (GRP78) was suppressed by nicotine in Tm-treated PC12 cells. Interestingly, the GRP78 expression was not changed by nicotine in Tg-treated cells. Moreover, nicotine reduced the activation of caspase-12 in Tm-treated cells, but not in Tg-treated cells. These results suggest that nicotine prevented Tm-induced ER stress-mediated apoptosis by attenuating an early stage of Tm-induced ER stress. It was possible that the suppression of GRP78 expression by nicotine was achieved through the suppression of the Ire1-XBP1 and/or ATF6 pathways. We observed that nicotine suppressed the Tm-induced, but not Tg-induced, splicing of XBP1 mRNA, and also suppressed the Tm-induced, but not Tg-induced, production of cleaved ATF6 in PC12 cells. These results indicate that the suppression of Ire1-XBP1 and ATF6 pathways contributes to the suppression of GRP78 expression by nicotine in Tm-treated PC12 cells, suggesting that nicotine suppresses a common step upstream of both the Ire1-XBP1 and ATF6 pathways which are required for the expression of GRP78 during Tm-induced ER stress.**

**Key words: apoptosis, ER stress, glucose-regulated protein 78, nicotine, PC12 cell.**

Abbreviations: ATF6, activating transcription factor 6; ER, endoplasmic reticulum; ERSE, ER stress response element; GRP78, glucose regulated protein 78; IRE1, inositol-requiring transmembrane kinase and endonuclease 1; JNK, c-Jun N-terminal kinase; Nup153, nucleoporin 153; PERK, PKR-like ER kinase; S1P, site 1 protease; S2P, site 2 protease; UPR, unfolded protein response; XBP1, X box-binding protein 1.

Chronic nicotine treatment blocks arachidonic acid-, glutamate-, β-amyloid- and tunicamycin (Tm)-induced neurotoxicity (*1*, *2*). Nicotine also promotes cell survival of spinal cord motorneurons (*3*). Many nicotinic acetylcholine receptor (nAChR) subtypes, for instance, α4β2 and α7, have been reported to be involved in these protective effects of nicotine (*4–8*). Calcium influx, activation of the PI3-kinase/Akt-mediated pathway, and induction of Bcl-2 have also been suggested to play important roles in nicotine-induced neuroprotective effects in various cells (*4*, *6*, *7*, *9–12*).

Neuronal degenerative diseases, including Alzheimer's, Parkinson's and polyglutamine diseases, are considered to involve endoplasmic reticulum (ER) stress, which leads to ER stress-mediated apoptosis (*13*). In the progress of these diseases, it has been reported that unfolded proteins are accumulated and that this step is critical in the progression of neuronal apoptosis through a caspase cascade involving an ER stress-specific caspase, caspase-12 or c-Jun N-terminal kinase (JNK) (*14*, *15*). In ER stress, unfolded proteins are accumulated in the ER lumen and accumulated unfolded proteins are sensitized by Ire1(α), ATF6 and PERK, leading to an unfolded protein response (UPR) (*16*, *17*). Ire1 generates spliced mRNA of XBP1 using its RNase activity, and then the XBP1 protein upregulates the expression of glucose-regulated protein 78 (GRP78), which works as a chaperone resident in the ER (*16*, *17*). ATF6 is cleaved by site 1 protease (S1P) and site 2 protease (S2P) to generate a p50ATF6 fragment that has transcriptional activity. Then, the p50ATF6 fragment upregulates the expression of GRP78 through an ER stress response element (ERSE) placed in the promoter region of the GRP78 gene (*16*, *17*). PERK has a kinase domain which phosphorylates the translational factor eIF2α, so *de novo* protein synthesis is prevented during ER stress, but the translation of some specific mRNAs including ATF4 is stimulated. The ATF4 protein, in turn, upregulates the expression of several genes implicated in the UPR, including CHOP/GADD153 (*16*, *17*). It is thought that all these processes are important in the accumulation attenuation of unfolded proteins during ER stress.

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Especially, functional analyses of Ire1 and ATF6 in the prevention of ER stress-induced apoptosis via upregulation of GRP78 expression have been reported (*16–19*).

ER stress can be chemically induced by Tm or thapsigargin (Tg). Tm induces ER stress by inhibiting the glycosylation of newly synthesized proteins and Tg induces ER stress by inhibiting $Ca^{2+}$-ATPase, which maintains $Ca^{2+}$ homeostasis in the ER. These compounds also lead to ER stress-mediated apoptosis via an intra-cellular mechanism activating the caspase cascade containing caspase-12 (*20*). At the same time, chaperone proteins, including GRP78, are upregulated during Tm- and Tg-induced ER stress. Thus, these two compounds activate the same intra-cellular signals involving ATF6, Ire1 and PERK described above to induce GRP78 expression, and trigger ER stress-mediated apoptosis when ER stress is severe. We previously reported that nicotine prevented Tm-induced ER stress-mediated apoptosis in PC12 cells, whereas it had no significant effect on Tg-induced apoptosis (*21*). Although the survival effect of nicotine on Tm-induced apoptosis might be mediated by calcium influx, activation of the PI3-kinase-Akt signalling pathway or induction of the Bcl-2 protein, the intra-cellular mechanism has not been fully elucidated. In the present study, we focused on the effect of nicotine on the expression of GRP78 in Tm- and Tg-treated PC12 cells. We found that nicotine downregulated the expression of GRP78 in Tm-treated cells, but not in Tg-treated cells. We also found that the activation of caspase-12 was suppressed by nicotine in Tm-treated cells, but not in Tg-treated cells. We observed that nicotine suppressed the Tm-induced, but not Tg-induced, splicing of XBP1 mRNA, and it also suppressed the Tm-induced, but not Tg-induced, production of cleaved ATF6 in PC12 cells. These results suggest that nicotine prevents Tm-induced ER stress-mediated apoptosis via the attenuation of an early stage, which is closely related to the activation of the Ire1-XBP1 pathway and ATF6 pathway, of Tm-induced ER stress, and that this protective mechanism does not work in Tg-treated cells.

## MATERIALS AND METHODS

*Reagents*—Nicotine (Nicotine tartrate dihydrate) was purchased from Nacarai. Tm, Tg, antibody against caspase-12, nifedipine, diltiazem and D-tubocurarine were purchased from Sigma. An anti-GRP78 antibody, an anti-HA probe (F-7) antibody and an anti-CHOP/GADD153 (R-20) antibody were purchased from SantaCruz. An anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from Chemicon. An anti-cleaved caspase-3 antibody was purchased from Cell Signaling, and an anti-Nup153 (nucleoporin 153) antibody from Covance. α-Bungarotoxin was obtained from Calbiochem.

*Cell Culture*—PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and 5% (v/v) heat-inactivated horse serum (HS). To measure cell viability, cells were seeded onto collagen coated 96-well plates at $1 \times 10^5$ cells/cm$^2$. The next day, the medium was changed to serum-free DMEM (SF-DMEM), and Tm, Tg or nicotine was added at this time point if necessary. After cultivation for 24 h, the cell viability was measured.

*Measurement of Cell Viability*—Viable cells were quantified by an MTT assay. Briefly, cells were seeded onto a 96-well plate and the medium was changed for the assay at an appropriate time. Then, the medium was changed again to SF-DMEM containing 5% (v/v) MTT solution and cells were incubated for 2 h. The fluorescence intensity was detected by a spectrophotometer at 750 nm. The cell viability was defined as [(test sample count) − (blank count)/(untreated control count) − (blank count)] × 100.

*Immunoblot Analysis*—PC12 cells were seeded onto 6-cm diameter dishes, and the next day the medium was changed to SF-DMEM containing Tm or Tg with or without nicotine. Then, cells were lysed in a lysis buffer as described previously (*22*). Total lysate (20 μg per lane) was loaded on SDS–PAGE and blotted onto a PVDF membrane using a semi-dry blotter (Atto). The primary antibody was loaded onto the membrane after blocking by skimmed milk (Nacarai) and a horseradish peroxidase-conjugated secondary antibody (SantaCruz) was reacted with it for 1 h. Bands were detected and visualized with a light capture system (Atto) using SuperSignal West Femto (Pierce).

*RT-PCR*—Total RNA was isolated using Isogen (Nippongene) and phenol–chloroform extraction procedure. cDNA was synthesized using First Strand cDNA Synthesis Kit (Amersham). The primers used were as follows: sense primer, 5′-AAACAGAGTAGCAGCGCAGA CTGC-3′ and anti-sense primer, 5′GGATCTCTAAAACTA GAGGCTTGGTG-3′ for XBP1; sense primer, 5′-TGGCAC AGTCAAGGCTGAGA-3′ and anti-sense primer, 5′CTTCT GAGTGGCAGTGATGG-3′ for GAPDH. The primer sets produce PCR products from unspliced XBP1 mRNA, its spliced form and GAPDH mRNA with a size of 600, 574 and 380 bp, respectively. PCR consisted of an initial denaturation cycle at 94°C for 2 min, followed by 30 cycles for GAPDH and 25 cycles for XBP1 of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and elongation at 68°C for 1min. An additional cycle at 72°C for 7 min completed the amplification process. Amplifed PCR products were separated by 2% for GAPDH and 3% for XBP1 agarose gel electrophoresis and visualized with ethidium bromide staining.

*Transfection of the Plasmid Expressing Human ATF6*—The expression plasmid pCGN-human-ATF6, hemagglutinin epitope (HA)-tagged full-length human ATF6 cDNA driven by the cytomegalo virus promoter 10, was kindly provided by Dr Ron Prywes (Department of Biological Science, Columbia University). PC12 cells grown to 50–60% confluence were transfected with the pCGN-human-ATF6 plasmid by lipofectamine 2000 (Invitrogen). The transfected cells were cultured for 48 h, and then the medium was changed to serum-free DMEM, and Tm, Tg or nicotine was added at this time point if necessary. After cultivation for an appropriate time, cells were collected. Nuclear fractions were extracted from whole cell lysates using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the manufacturer's instructions.

*Statistical Evaluation*—All values in the figure are expressed as the mean ± SEM. The Student's *t*-test or one-way ANOVA was used to compare means between groups. A *P*-value of $< 0.05$ was considered statistically significant.

## RESULTS

We first compared the survival effect of nicotine on PC12 cells treated with Tm and its effect on those treated with Tg. It is well known that Tm and Tg induce ER stress-mediated apoptosis in various cells. We found that nicotine markedly prevented Tm-induced ER stress-mediated apoptosis, but not Tg-induced ER stress-mediated apoptosis (Fig. 1). Nakagawa *et al*. (*14*) reported that the activation of caspase-12, which is known as an ER stress-specific caspase, was involved in the progression of ER stress-mediated apoptosis. We previously reported that caspase-12 was activated during both Tm- and Tg-induced apoptosis in PC12 cells, and that zVAD-fmk, a general caspase inhibitor, suppressed the activation of caspase-12 induced by both Tm and Tg to the control level (*22*, *23*). To examine the blocking of ER stress-mediated apoptosis progression by nicotine, we measured the activation of caspase-12 by detecting the bands of active fragments as reported by Nakagawa *et al*. (*14*). As shown in Fig. 2, the activation of caspase-12 was decreased by nicotine in Tm-treated cells, but not in Tg-treated cells. These results indicate that nicotine prevents the progression of only Tm-induced ER stress-mediated apoptosis.

Caspase-3 is one of the executer caspases of all types of apoptosis. It has been reported that caspase-3 catalyses the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), a 116 kDa nuclear enzyme closely linked to apoptosis. Because the activation of caspase-12 leads to the activation of caspase-3 (Fig. 3), which cleaves PARP, we examined whether nicotine could inhibit the Tm-induced cleavage of PARP and, as a control, whether zVAD-fmk could also prevent the Tm-induced cleavage of PARP. As shown in Fig. 4, treatment with 1 μg/ml Tm or 0.3 μM Tg caused the cleavage of PARP, and 5 μM nicotine inhibited the Tm-induced, but not Tg-induced, cleavage of PARP. As a control, zVAD-fmk prevented the Tm-induced cleavage of PARP (data not shown). These results confirmed that nicotine prevents the total progression of only Tm-induced ER stress-mediated apoptosis.

It has been reported that the expression of glucose-regulated protein 78 (GRP78) is upregulated during ER stress and that GRP78 has a protective effect against ER stress (*16–18*). To elucidate the molecular mechanism of nicotine prevention, we analysed the expression of GRP78 for 24 h after the addition of nicotine to Tm- and Tg-treated cells by immunoblotting. Interestingly,

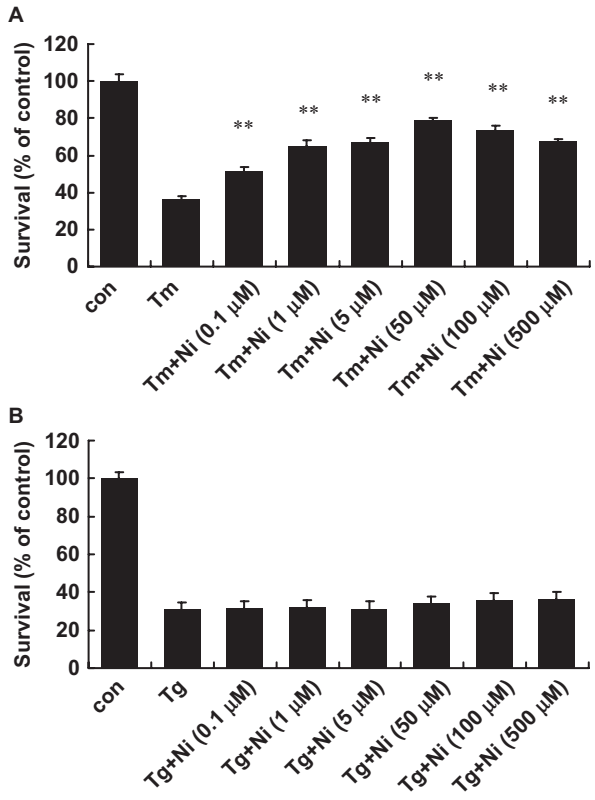


Fig. 1. **Survival effect of nicotine on Tm-induced ER stress-mediated apoptosis.** (A and B) PC12 cells were untreated (con) or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) as described in MATERIALS AND METHODS section. The final concentrations of nicotine are shown in parentheses. PC12 cells were maintained for 24 h, and viable cells were quantified using an MTT assay. Values are means ± SEM ($n = 4$) and statistical analysis was carried out with one-way ANOVA. $^{**}P < 0.001$.

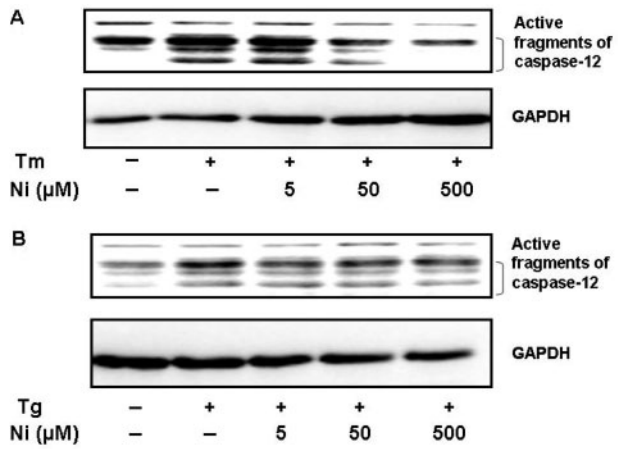


Fig. 2. **Nicotine suppresses activation of caspase-12 in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 24 h were lysed in lysis buffer as described in MATERIALS AND METHODS section. Twenty micrograms of total protein per lane were used for SDS–PAGE. Then, immunoblotting was carried out using an anti-caspase-12 antibody (Sigma) or anti-GAPDH antibody (Chemicon). The bands were visualized with a light capture system and the location of active fragments of caspase-12 is shown on the right. The results are representative of eight independent experiments.

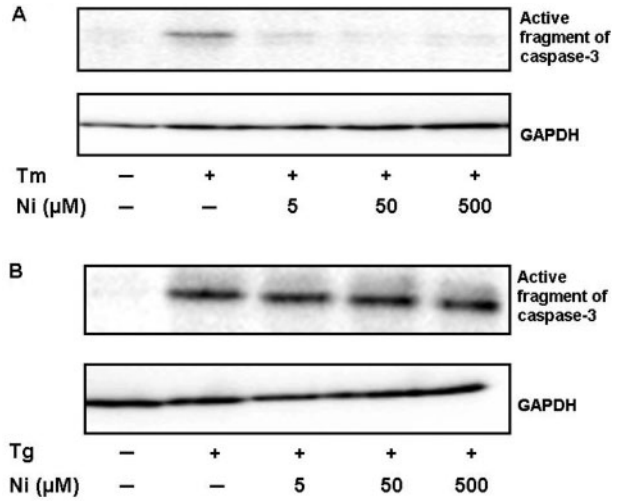


Fig. 3. **Nicotine suppresses activation of caspase-3 in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 24 h were lysed in lysis buffer as described in MATERIALS AND METHODS section. Twenty micrograms of total protein per lane were used for SDS–PAGE. Then, immunoblotting was carried out using an anti-cleaved caspase-3 antibody (Cell Signalling) or anti-GAPDH antibody (Chemicon). The results are representative of five independent experiments.

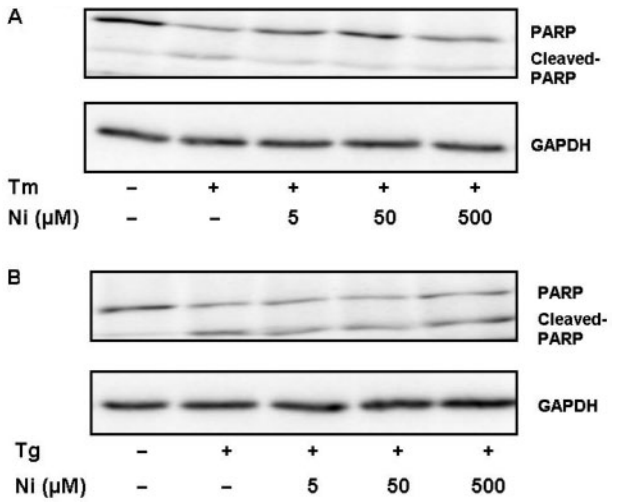


Fig. 4. **Nicotine suppresses cleavage of PARP in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 24 h were lysed in lysis buffer as described in MATERIALS AND METHODS section. Twenty micrograms of total protein per lane were used for SDS–PAGE. Then, immunoblotting was carried out using an anti-PARP antibody (Cell Signaling) or anti-GAPDH antibody (Chemicon). The results are representative of three independent experiments.

nicotine suppressed the expression of GRP78 in Tm-treated cells (Fig. 5). However, the expression of GRP78 was not altered by nicotine in Tg-treated cells. These data indicate that Tm-induced ER stress-mediated

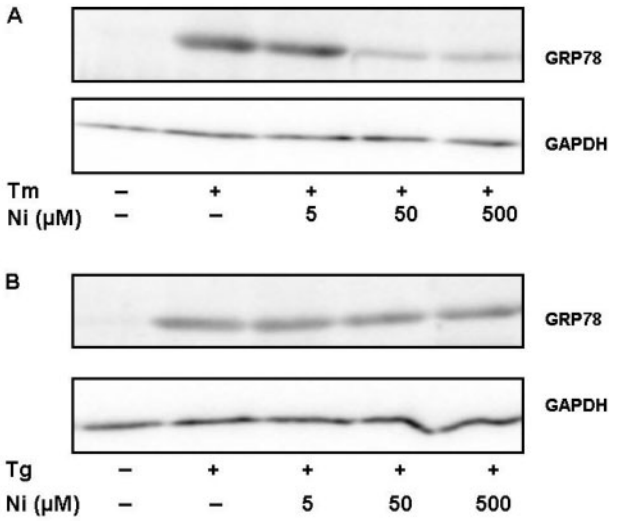


Fig. 5. **Nicotine suppresses GRP78 expression in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 24 h were lysed in lysis buffer as described in MATERIALS AND METHODS section. Immunoblotting was carried out using an anti-GRP78 antibody (SantaCruz) or anti-GAPDH antibody (Chemicon). The bands were visualized. The results are representative of nine independent experiments.

apoptosis is suppressed by nicotine through the attenuation of an early stage of Tm-induced ER stress. As mentioned above, Tm inhibits the glycosylation of newly synthesized proteins and then initiates ER stress by accumulating unglycosylated unfolded proteins in the ER. On the other hand, Tg disturbs $Ca^{2+}$ homeostasis in the ER and then initiates ER stress by an unknown mechanism leading to the accumulation of unfolded proteins in the ER. The ER stress-attenuating effect of nicotine may be related to the difference between the two initial mechanisms of ER stress which are induced by Tm and Tg.

Next, we examined the effects of antagonists of nAChR and L-VSCC (L-type voltage-sensitive $Ca^{2+}$ channel) on the nicotine suppression of Tm-induced GRP78 expression, as well as a Tm-induced apoptosis (*21*). As shown in Fig. 6, L-VSCC antagonists (nifedipine and diltiazem) and a non-selective nicotine antagonist, D-tubocurarine, completely inhibited the effect of nicotine on the Tm-induced GRP78 expression. α-Bungarotoxin which antagonizes α7-type nAChR also inhibited the suppressive effect of nicotine on the Tm-induced GRP78 expression. We previously showed that although α-bungarotoxin antagonized the nicotine-induced calcium influx at lower nicotine concentrations, the toxin had no effect on nicotine-induced phosphorylation of ERK, CREB and Akt in PC12 cells (*21*). Taken together, both α7-type and non-α7-type nAChR are likely to be involved in the nicotine-induced suppression of Tm-induced GRP78 expression in PC12 cells.

The *GRP78* gene has a *cis*-element ERSE in the promoter region (*16*, *17*). During ER stress, cleaved ATF6 works as a transcriptional factor binding to ERSE

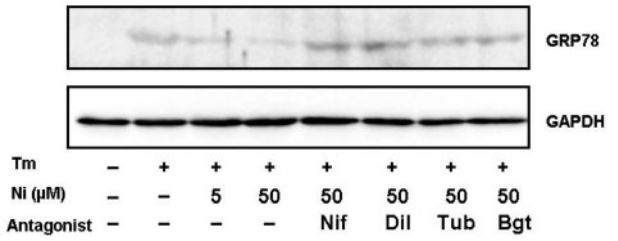


Fig. 6. **Effects of antagonists of nAChR and L-VSCC on the nicotine suppression of the Tm-induced GRP78 expression.** PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) in the absence or presence of nicotine (Ni) without or with 10 μM nifedipine (Nif), 10 μM diltiazem (Dil), 0.1 μM D-tubocurarine (Tub) or 0.1 μM α-bungarotoxin (Bgt) for 24 h were lysed in lysis buffer as described in MATERIALS AND METHODS section. Immunoblotting was carried out using an anti-GRP78 antibody (SantaCruz) or anti-GAPDH antibody (Chemicon). The bands were visualized as described in MATERIALS AND METHODS section. The results are representative of three independent experiments.

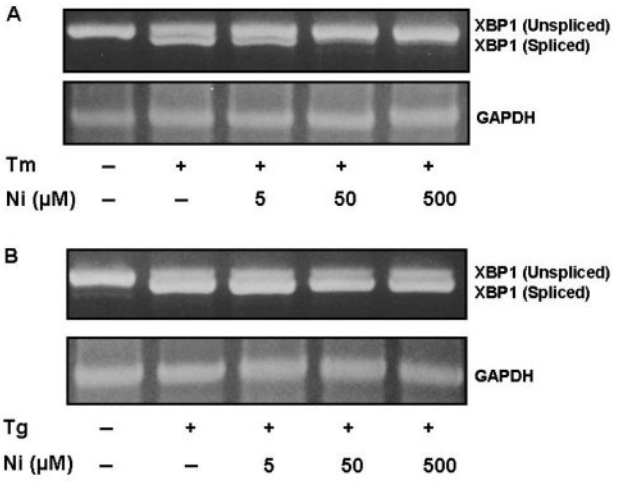


Fig. 7. **Effect of nicotine on XBP1 mRNA splicing in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 4 h (A) or 2 h (B) were lysed. Total RNA was isolated using Isogen and a phenol–chloroform extraction procedure, and RT-PCR was carried out as described in MATERIALS AND METHODS section. The results are representative of four independent experiments.

to induce GRP78 expression. Moreover, XBP1, which is generated from spliced XBP1 mRNA by Ire1, also binds to ERSE to induce GRP78 expression (*16*, *17*). It is possible that the nicotine suppression of GRP78 expression is achieved through the suppression of the Ire1-XBP1 and ATF6 pathways.

Therefore, we examined the splicing of XBP1 mRNA and the cleavage of ATF6 during Tm- or Tg-induced ER stress and the effect of nicotine on these events. First, we performed an RT-PCR assay to detect the spliced form of XBP1 in Tm- or Tg-treated PC12 cells. As shown in Fig. 7, although both Tm and Tg caused the splicing of XBP1 mRNA during ER stress, nicotine suppressed only the Tm-induced splicing of XBP1 mRNA. Second, we

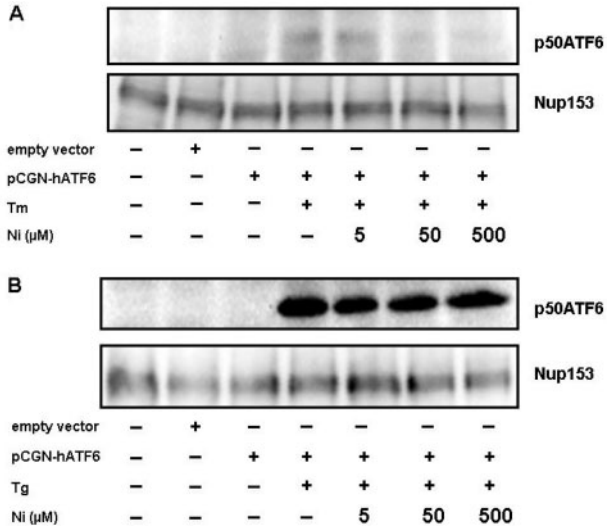


Fig. 8. **Nicotine inhibits cleavage of ATF6 in Tm-treated cells.** (A and B) PC12 cells were transfected with control plasmid pCGN (empty vector) or the expression plasmid pCGN-human-ATF6 (pCGN-hATF6). PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 6 h (A) or 4 h (B) were lysed. Each nuclear fraction was extracted from whole-cell lysates using the NE-PER Nuclear and Cytoplasmic Extraction Reagents as described in MATERIALS AND METHODS section. Twenty micrograms of total protein per lane were used for SDS–PAGE. Then, immunoblotting was carried out using an anti-HA probe (F-7) antibody (SantaCruz) or anti-Nup 153 antibody (Covance). The results are representative of five independent experiments.

performed immunoblot analysis using an anti-HA antibody to detect ectopically expressed HA-tagged human ATF6 in Tm- or Tg-treated PC12 cells. Both Tm and Tg caused the cleavage of ectopically expressed human ATF6 (Fig. 8). This result showed that the expressed human ATF6 was correctly translocated into the ER membrane in PC12 cells. In the nicotine effect on the splicing of XBP1 mRNA, nicotine could suppress the Tm-induced, but not Tg-induced, production of cleaved ATF6 in PC12 cells (Fig. 8).

In addition, we performed immunoblot analysis using an anti-CHOP/GADD153 antibody to detect the CHOP expression in Tm- or Tg-treated PC12 cells. As shown in Fig. 9, although both Tm and Tg induced the expression of CHOP protein during ER stress, nicotine suppressed only the Tm-induced expression of CHOP protein.

## DISCUSSION

ATF6 is a key transcriptional factor of the unfolded protein response (UPR) during ER stress. ATF6, a 90 kDa ER transmembrane protein, contains three evolutionarily conserved N-linked glycosylation sites within its carboxyl terminal domain. The activation of p90ATF6 requires a transit from the ER to the Golgi apparatus, where it is cleaved by the S1P/S2P protease system to generate a nuclear form, p50ATF6, which acts as a transcriptional factor. The amount of p50ATF6 in a nuclear fraction of Tm-treated PC12 cells was decreased

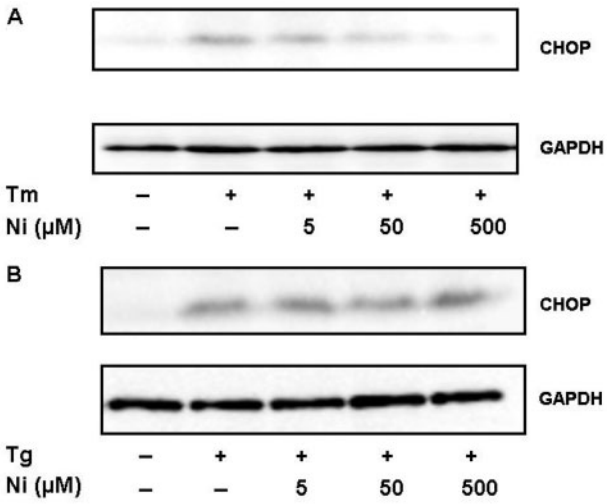


Fig. 9. **Nicotine suppresses CHOP expression in Tm-treated cells.** (A and B) PC12 cells untreated or treated with 1 μg/ml tunicamycin (Tm) (A) or 0.3 μM thapsigargin (Tg) (B) in the absence or presence of nicotine (Ni) for 6 h (A) or 4 h (B) were lysed. Immunoblotting was carried out using an anti-CHOP/GADD153 (R-20) antibody (SantaCruz) or anti-GAPDH antibody (Chemicon). The bands were visualized as described in MATERIALS AND METHODS section. The results are representative of two independent experiments.

by nicotine (Fig. 8). During Tg-induced ER stress, the amount of p50ATF6 did not change in the presence of nicotine.

The processing of ATF6 was a little slower in Tm-treated cells than that in Tg-treated cells, but p50ATF6 appeared at nearly the same time as the increase in the level of GRP78 mRNA (data not shown), suggesting that the proteolytic conversion of p90ATF6 to p50ATF6 is a key regulatory step in GRP78 expression. As shown in Fig. 8, the processing of ATF6 was significantly weaker in Tm-treated cells than that in Tg-treated cells, suggesting that nicotine could inhibit the weak processing of ATF6 induced by Tm but not the strong processing of ATF6 induced by Tg.

The data shown in Fig. 7 suggest that the suppression of Ire1-XBP1 pathway also contributes to the suppression of GRP78 expression by nicotine in Tm-treated PC12 cells, because nicotine had no effect on the splicing of XBP1 mRNA in Tg-treated PC12 cells. The splicing of XBP1 mRNA was significantly weaker in Tm-treated cells than that in Tg-treated cells (Fig. 7), suggesting that nicotine could inhibit the weak activation of the Ire1-XBP1 pathway induced by Tm, but not the strong activation of the Ire1-XBP1 pathway induced by Tg. We consider that both the Ire1-XBP1 pathway and the ATF6 pathway are required for the expression of GRP78 during ER stress (*18*, *24*). We also consider that nicotine suppresses a common and weak step upstream of both the Ire1-XBP1 and ATF6 pathways which are required for the expression of GRP78 in Tm-treated PC12 cells, but that nicotine could not suppress a common and strong step in Tg-treated PC12 cells. The action of nicotine might have an upper limit to show a protective effect against ER stress. The common step upstream of both the Ire1-XBP1 and ATF6 pathways in Tm-treated PC12 cells is also considered to be upstream of the PERK(-eIF2α-ATF4-CHOP) pathway (Fig. 9). This common step may be closely related to the accumulation of unfolded proteins in the ER during ER stress.

As shown in Figs 2 and 3, the activation of caspase-12 and -3, which should occur after the accumulation of unfolded proteins, was also suppressed by nicotine in addition to the suppression of GRP78 expression in Tm-treated cells. Tg is an inhibitor of $Ca^{2+}$-ATPase, which is located on the ER membrane, and disturbs $Ca^{2+}$ homeostasis in the ER. If nicotine affects the $Ca^{2+}$-mediated cell survival mechanism in the ER, it could explain why nicotine does not have a cell survival effect on Tg-induced ER stress. Although 50 μM nicotine effectively downregulated the Tm-induced GRP78 expression as 500 μM nicotine (Fig. 5), it weakly suppressed the activation of caspase-12 by Tm in comparison with 500 μM nicotine (Fig. 2). This discrepancy may be explained by the partial contribution of caspase-12 to the progression of ER stress-induced apoptosis. We consider that there may be some additional progression mechanisms of ER stress-induced apoptosis other than the activation of caspase-12. In addition, although 5 μM nicotine completely downregulated the activation of caspase-3 (Fig. 3), it weakly suppressed the Tm-induced expression of GRP78 (Fig. 5). We also consider that nicotine may have some additional protection mechanisms against ER stress-induced apoptosis other than the suppression of ER stress itself as described below.

Kane *et al*. (*25*) showed that exposure to nicotine increases mRNA and protein levels of proteasomal components in rat prefrontal cortical (PFC) neurons. The upregulation of genes encoding the ubiquitin/proteasome system (UPS) components by nicotine might represent a feedback mechanism attempting to counteract the proteasomal inhibition (*26*). Nicotine is known to affect brain function by binding to nAChRs and via the newly discovered interaction with the UPS (*26*). The interaction of nicotine with the UPS might underlie the ability of nicotine to both produce addiction and enhance cognition. The ubiquitin/proteasome pathway targets unfolded proteins for degradation, and ER chaperones such as GRP78 are involved in the folding of unfolded proteins. These multiple pathways may crosstalk during ER stress. The implicated nicotine function as an important modulator of cellular homeostasis suggests that nicotine has an important role in other homeostasis pathways such as those of protein modification and degradation (*26*). Nicotine-induced inhibition of UPS has been suggested to regulate numerous postsynaptic proteins by ubiquitination in response to synaptic activity in central nervous system (CNS) neurons. The nicotine-induced direct inhibition of UPS might participate in nicotine-dependent synaptic plasticity (*26*) and possibly in neuronal protection.

It has been reported that Akt, which is known as a survival-promoting kinase, is involved in the nicotine-mediated signalling pathway through nAChRs (*27*). We should focus on the signalling pathway induced by nicotine in relation to Akt to clarify the mechanism of the survival effect of nicotine on Tm-induced ER stress.

Further studies are necessary to elucidate the detailed mechanism of nicotine action on Tm-induced ER stress.

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